

Pontin and Reptin regulate cell proliferation in early *Xenopus* embryos in collaboration with c-Myc and Miz-1

Christelle Etard^{a,1,2}, Dietmar Gradl^{a,2}, Martin Kunz^a, Martin Eilers^b, Doris Wedlich^{a,*}

^aZoologisches Institut II, Universität Karlsruhe (TH), Kaiserstrasse 12, 76131 Karlsruhe, Germany

^bInstitut für Molekularbiologie und Tumorforschung (IMT), Universität Marburg, 35033 Marburg, Germany

Received 12 January 2004; received in revised form 17 November 2004; accepted 17 November 2004

Available online 10 December 2004

Abstract

Pontin (Tip49) and Reptin (Tip48) are highly conserved components of multimeric protein complexes important for chromatin remodelling and transcription. They interact with many different proteins including TATA box binding protein (TBP), β -catenin and c-Myc and thus, potentially modulate different pathways. As antagonistic regulators of Wnt-signalling, they control wing development in *Drosophila* and heart growth in zebrafish. Here we show that the *Xenopus* xPontin and xReptin in conjunction with c-Myc regulate cell proliferation in early development. Overexpression of xPontin or xReptin results in increased mitoses and bending of embryos, which is mimicked by c-Myc overexpression. Furthermore, the knockdown of either xPontin or xReptin resulted in embryonic lethality at late gastrula stage, which is abrogated by the injection of c-Myc-RNA. The N-termini of xPontin and xReptin, which mediate the mitogenic effect were mapped to contain c-Myc interaction domains. c-Myc protein promotes cell cycle progression either by transcriptional activation through the c-Myc/Max complex or by repression of cyclin dependent kinase inhibitors (p21, p15) through c-Myc/Miz-1 interaction. Importantly, xPontin and xReptin exert their mitogenic effect through the c-Myc/Miz-1 pathway as dominant negative Miz-1 and wild-type c-Myc but not a c-Myc mutant deficient in Miz-1 binding could rescue embryonic lethality. Finally, promoter reporter studies revealed that xPontin and xReptin but not the N-terminal deletion mutants enhance p21 repression by c-Myc. We conclude that xPontin and xReptin are essential genes regulating cell proliferation in early *Xenopus* embryogenesis through interaction with c-Myc. We propose a novel function of xPontin and xReptin as co-repressors in the c-Myc/Miz-1 pathway.

© 2004 Elsevier Ireland Ltd. All rights reserved.

Keywords: Cyclin dependent kinase inhibitors; Transcription complex; Tip49; Tip 48; β -Catenin; Axis formation

1. Introduction

Pontin (Tip49) and Reptin (Tip48) are evolutionary conserved proteins that are present in all eukaryotes analysed so far (Kanemaki et al., 1999; Bauer et al., 2000; Lim et al., 2000; Wood et al., 2000, Etard et al., 2000, Rottbauer et al., 2002). Disruption of the yeast homologues of Pontin and Reptin results in both enhanced and reduced

expression of multiple genes suggesting that Pontin and Reptin may function both in gene activation and repression (Jonsson et al., 2001). They also play important roles in developmental processes as they function as antagonistic regulators of Wnt-signalling by binding to β -catenin. The zebrafish mutant *liebeskummer* (*lik*) was identified as a 9 bp insertion in the *zReptin* gene, which results in an aberrant splice form of *zReptin*. In *lik* animals, the co-repressor function of *zReptin* in β -catenin/Tcf signalling is increased and leads to hyperplastic growth of the heart (Rottbauer et al., 2002). In *Drosophila*, *dPontin* and *dReptin* contribute to wing development. Loss of function of these genes strongly modified wing phenotypes with genetically altered armadillo expression (Bauer et al., 2000). As maternally provided proteins, Pontin and Reptin are present throughout

* Corresponding author. Tel.: +49 721 608 3990; fax: +49 721 608 3992.

E-mail address: doris.wedlich@zi2.uni-karlsruhe.de (D. Wedlich).

¹ Institut de Génétique et de Biologie Moléculaire et Cellulaire, 1 rue Laurent Fries, 67404 Illkirch Cedex C.U. de Strasbourg, France.

² These authors contributed equally to this work.

embryogenesis (Bauer et al., 2000; Etard et al., 2000; Rottbauer et al., 2002). They also seem to play a crucial role in early development because flies zygotically deficient for *dPontin* or *dReptin* die at first instar without any Wg/Wnt phenotype (Bauer et al., 2000). Furthermore, injections of zReptin antisense oligonucleotide morpholino in fish embryos reveal early and pleiotropic defects (Rottbauer et al., 2002). These observations point to additional functions of Pontin and Reptin that are independent of the Wnt/ β -catenin pathway.

Initially, the homologues of Pontin and Reptin in human, rat and yeast (TIP49/TIP49a and TIP49b/TIP48) were characterized to interact with TATA-box binding protein (TBP) (Kanemaki et al., 1997; Kanemaki et al., 1999; Makino et al., 1999; Wood et al., 2000). They all share the conserved domains Walker A and Walker B. These motifs are also found in the RuvB bacterial recombination factor and have been characterized to bind (Walker A) and hydrolyze ATP (Walker B) (Hishida et al., 1999). The ATP dependent DNA-helicase RuvB is a component of the DNA inducible repair system and catalyses branch migration in holiday junctions (Shinagawa and Iwasaki, 1996). Biochemical investigations also demonstrated that the rat homologue TIP49a of Pontin, possesses ATPase activity stimulated by the presence of single stranded DNA and that it is able to unwind DNA duplex (Makino et al., 1999). The enzymatic properties of Pontin and Reptin are the same except for the polarity of the DNA-helicase activity (Kanemaki et al., 1999). DNA-stimulated ATPase activity has also been demonstrated for zReptin and was increased in the *lik* mutant (Rottbauer et al., 2002).

Importantly, Pontin and Reptin were found complexed with c-Myc in vivo and functionally coupled to its oncogenic activity (Wood et al., 2000). Depending on the composition of transcription complexes c-Myc is able to activate and/or to repress target genes (reviewed by Eisenman, 2001; Wanzel et al., 2003). By forming heterodimers with the bHLH transcription factor Max, c-Myc directly binds to E-boxes and activates cell cycle genes like cyclinD2 (Bouchard et al., 2004). Alternatively, c-Myc can bind to the zinc finger transcription factor Miz-1 and block Miz-1 target gene expression. In the latter transcription complex Miz-1 but not c-Myc binds to specific DNA sequences. Target genes of the repressive c-Myc activity are the cyclin dependent kinase inhibitor proteins p21 and p15 (Staller et al., 2001; Seoane et al., 2001; 2002; Herold et al., 2002). Thus, transcriptional activation and repression by c-Myc results in an increase in cell proliferation. In *Xenopus* embryos c-Myc is the only member of the multi-gene family that is provided maternally (Bellmeyer et al., 2003). The maternal and the post-gastrula c-Myc are encoded by two different genes. Only one of them, c-Myc I, is expressed from the zygotic genome in post-gastrula embryos (Vriz et al., 1989). Recently, a function of *Xenopus* c-Myc I in neural crest induction has been reported, which is not coupled to cell proliferation (Bellmeyer et al., 2003).

To investigate the function of xPontin and xReptin in early *Xenopus* development, we performed gain-of function experiments with wild-type and mutant forms of both proteins. We found that *Xenopus* Pontin and Reptin failed to influence dorsoventral axis formation. Instead, overexpression of either xPontin or xReptin resulted in increased cell proliferation, giving rise to convex or concave shaped phenotypes depending on the site of injection. On the contrary, knockdown using antisense morpholino oligonucleotides led to reduced mitoses and, at higher concentrations, to embryonic lethality. Expression of c-Myc and a dominant negative Miz-1 mutant but not a c-Myc mutant deficient in Miz-1 binding were able to rescue the morpholino phenotypes. This indicates that xPontin and xReptin collaborate with c-Myc/Miz-1 in controlling cell proliferation in *Xenopus* development. Furthermore, mutation analyses, co-precipitation experiments and promoter reporter assays revealed that the N-termini of both xPontin and xReptin are essential for c-Myc binding, p21 repression and mitogenic function.

2. Results

2.1. xPontin and xReptin overexpression increases cell proliferation

We initially expressed the full-length proteins and different mutants of *Xenopus* Pontin and Reptin (Fig. 1A) in 4-cell stage embryos to study whether these proteins might modulate the canonical Wnt/ β -catenin signalling pathway. These experiments led to negative results since neither xPontin nor xReptin or any of the mutants affected axis formation or altered the expression of *Siamois* and *Xnr3* (Supplementary Fig. S1). Instead, we observed a bending of embryos, which could be correlated to increased cell proliferation. Overexpression of either xPontin or xReptin results in identical phenotypes: a convex or concave bending of embryos, depending on the blastomeres, which are injected (dorsal versus ventral, Fig. 1B). The convex shape of dorsally injected embryos occurred more frequently than the concave phenotype typical for ventral injections (dorsal: ventral ratio: Pontin 42%:20%; Reptin 44%:12%). To examine a possible influence of the conserved Walker A and B motifs, we tested the mutant constructs depicted in Fig. 1A for their ability to induce a bent phenotype. Deletion of single Walker motifs did not affect the frequency of bent phenotypes (Fig. 1C). In contrast, deletion of the N-terminus abrogated the ability of either protein to induce bending of embryos (Fig. 1C). Therefore, we conclude that the N-termini but not the Walker motifs play a crucial role for xPontins and xReptins function in early embryogenesis.

The bent phenotype may reflect an increase in cell proliferation at the injected side, which led to a single-sided elongation of the embryo. This idea was supported by

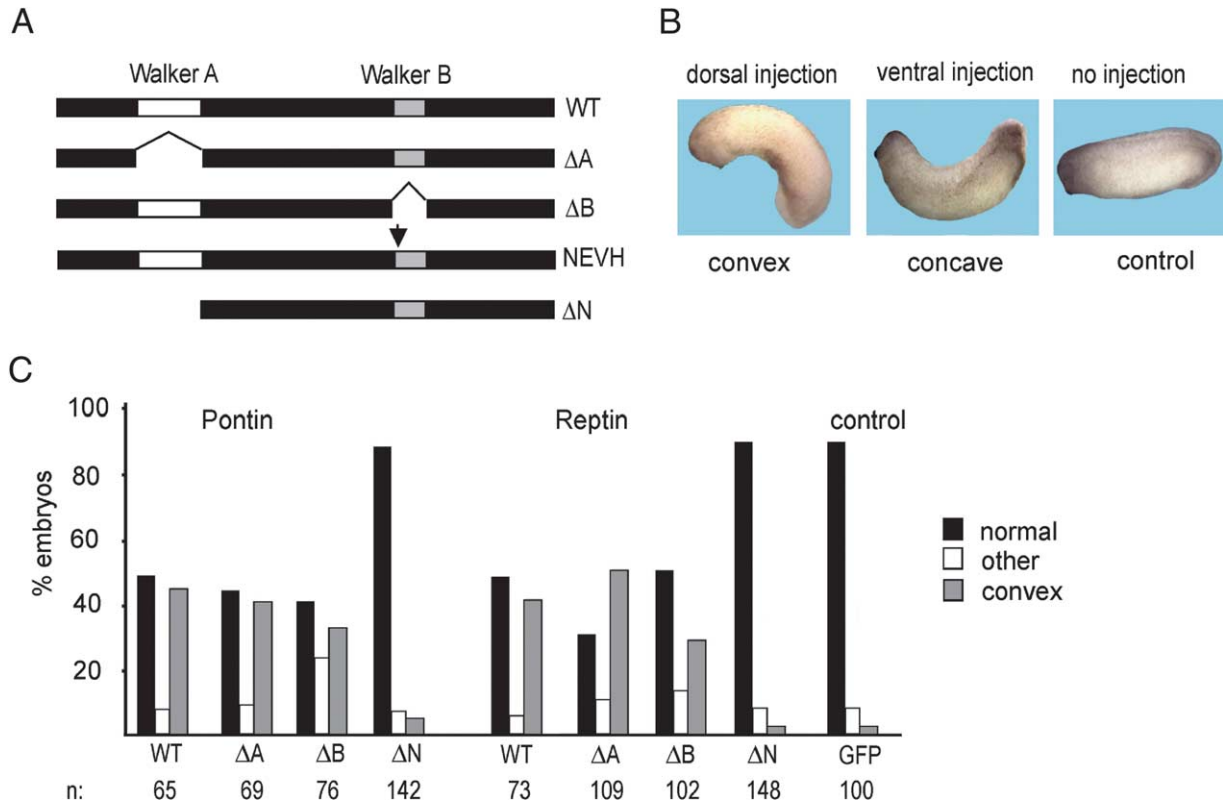


Fig. 1. xPontin and xReptin overexpression result in bending of *Xenopus* embryos. (A) Diagram of *Xenopus* wtPontin (457aa) and wtReptin (462aa) and the mutants used. The position of the Walker A (white box) and the Walker B (grey box) motifs are indicated. Point mutants "NEVH" were generated according to (Mezard et al., 1997): in Pontin this is D302N, in Reptin D299N. N-terminal deletions are Pontin Δ N: Δ 1–135aa, Reptin Δ N: Δ 1–84aa. Walker A deletion: Pontin Δ 70–77, Reptin Δ 76–83, Walker B deletion: Pontin Δ 302–306, Reptin Δ 299–303. All constructs contain a myc-tag at the C-terminus. (B) Overexpression of xPontin and xReptin induces bending of embryos. Dorsal injections led to convex, ventral injections to concave shaped embryos. XPontin injected embryos are exemplarily shown. (C) Percentage of embryos displaying a convex phenotype upon injection of the indicated constructs into both dorsal blastomeres of *Xenopus* 4-cell stage embryos. Phenotypes were scored at stage 30 (Nieuwkoop and Faber, 1967). The numbers of injected embryos are given at the bottom.

the finding that bending was abolished when xPontin overexpressing embryos were treated with hydroxyurea and aphidicolin (HUA), which blocks cell divisions (Fig. 2A). To further confirm the results we quantified cell proliferation in sections of injected embryos immunostained for phosphohistone H3 (PH3), a marker of mitotic cells. Embryos were injected with full-length, Walker A and Walker B deleted as well as N-terminally truncated xPontin and xReptin mRNA into one blastomere at the 2-cell stage. Coinjected GFP-RNA served to trace the injected side. Overexpression of xPontin resulted in an increased number of PH3 positive nuclei at stage 12.5 (Fig. 2B and supplementary Fig. S2, for stage classification see Nieuwkoop and Faber, 1967) while at early gastrula (stage 10.5), no significant difference in proliferation was found (Fig. 2B). This was also observed for xReptin but was less pronounced (Fig. 2B). Deletion mutants lacking the Walker A or Walker B motif increased cell divisions as full-length xPontin (Fig. 2B). Importantly, deletion of the N-terminus in xPontin and xReptin abolished this effect (Fig. 2B). At stage 20 this difference in stimulating cell

proliferation between the full-length and the N-terminal truncated proteins was still obvious (data not shown).

2.2. Knockdown of either xPontin or xReptin leads to embryonic lethality

We then asked whether xPontin or xReptin are required for cell proliferation. For this purpose, antisense morpholino oligonucleotides against xPontin (PMo) and xReptin (RMo) were designed. Corresponding morpholino oligonucleotides with 4 mismatched bases were generated as control (CoMo). Antisense, but not control morpholino oligonucleotides efficiently blocked translation of exogenously expressed xPontin and xReptin (Fig. 3A). Cell proliferation was again quantified by PH3 immunostaining. A significant decrease of mitotic cells was detected upon injection of the antisense morpholino oligonucleotides (Fig. 3B). Injection of the control morpholino oligonucleotides had no effect (Fig. 3B and supplementary Fig. 2). The reduction in cell proliferation by PMo and RMo was already seen at early gastrula (stage 10.5) and increased in

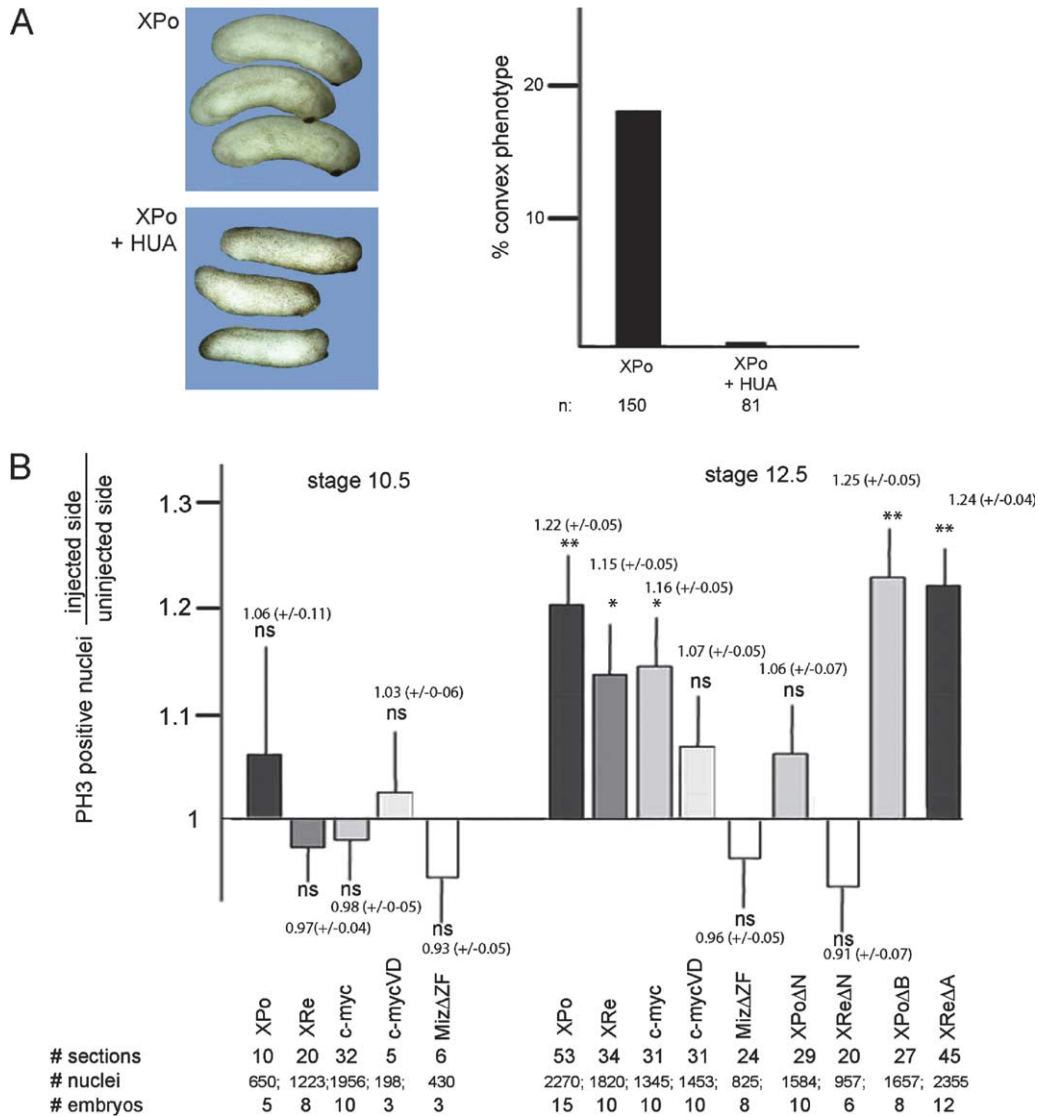


Fig. 2. xPontin and xReptin induce hyperproliferation in *Xenopus* embryos (A) Treatment of Pontin injected embryos with 20 mM hydroxyurea and 150 μ M aphidicolin (+ HUA, inhibitors of cell division) resulted in a loss of bent phenotypes. Graph: Quantification of bent phenotypes of hydroxyurea and aphidicolin (+ HUA) treated embryos. (B) Quantification of the PH3 positive nuclei at stage 10.5 and 12.5. Values are given in relation to the non-injected side. 1 ng mRNA of the indicated construct was co-injected with 50 pg GFP mRNA into one blastomere of two-cell stage embryos. The PH3 positive nuclei of the non-injected side were taken as 1. ***p*-value in student *t*-test <0.005, * *p*-value in student *t*-test <0.02, 'ns' *p*-value in student *t*-test >0.02. XPo: xPontin, XRe: xReptin.

later stages (12.5). When both blastomeres were injected with antisense morpholino oligonucleotides nearly 50% of the embryos showed a developmental arrest at late gastrula stage followed by embryonic lethality. At that time the remaining embryos and the controls reached neurula stage and developed normally (Fig. 3C,D). As also seen in Fig. 3D co-injection of mRNA encoding human Pontin or Reptin together with the corresponding antisense morpholino rescued embryonic lethality. hReptin was less efficient in the rescue experiment than hPontin. Interestingly, expression of xPontin did not rescue the embryonic lethality induced by ablation of xReptin. A partial rescue was observed with xReptin in xPontin depleted embryos (Fig. 3D). This indicates that both proteins cannot

completely replace each other. Co-injection of the N-terminally deleted proteins with the corresponding antisense morpholino led to strongly reduced embryonic survival, suggesting that translational suppression of endogenous xPontin and xReptin exposes a dominant negative character of both N-terminal deletion mutants.

2.3. Overexpression or depletion of xPontin and xReptin did not alter mesodermal marker gene expression

Embryonic lethality is a dramatic phenotype, which apart from blocking cell proliferation could also result from inhibition of gene transcription. Therefore, we analysed the expression of mesodermal marker genes, which are

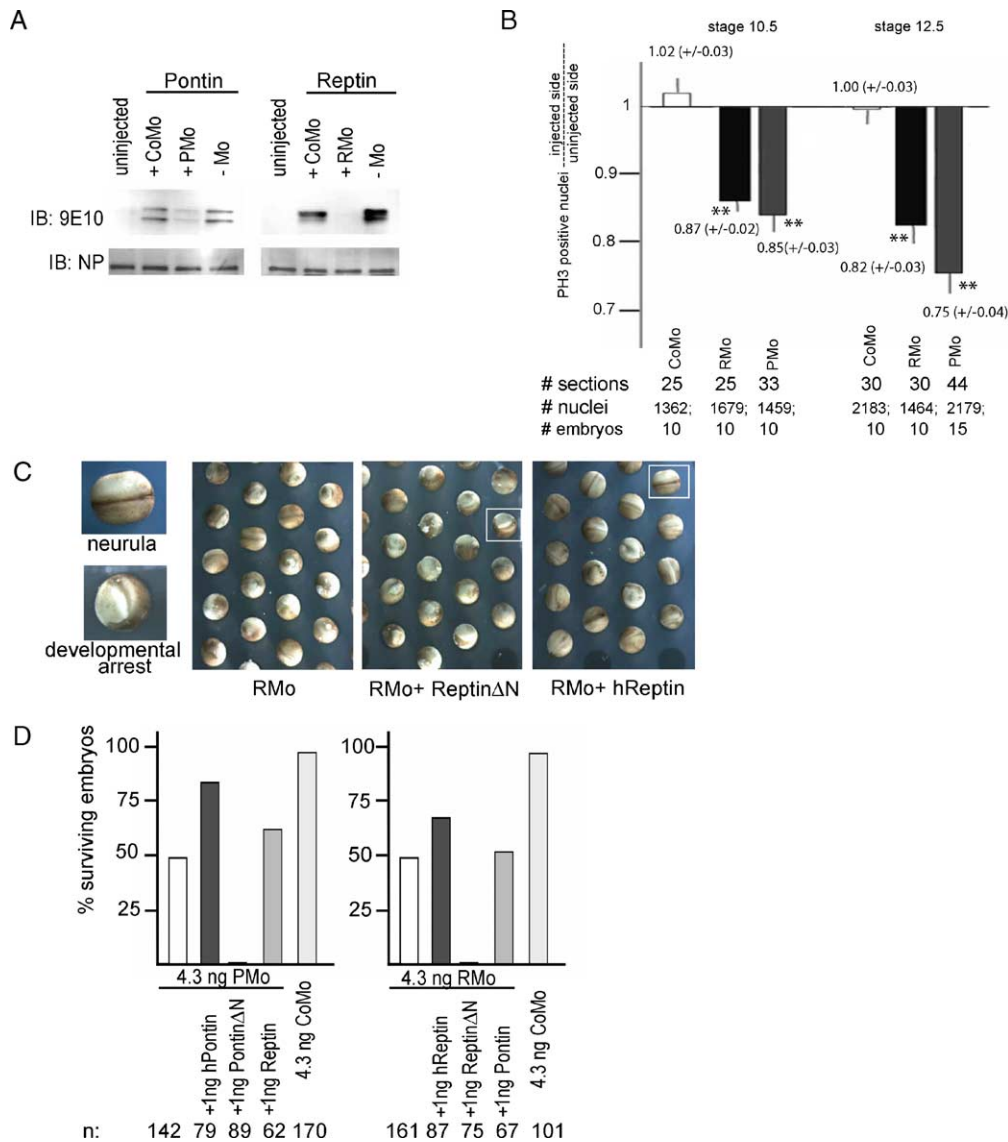


Fig. 3. xPontin and xReptin are required for proliferation and development beyond the gastrula stage in *Xenopus* embryos (A) Immunoblots documenting expression of xPontin and xReptin in early neurulae upon injection of the indicated morpholino oligonucleotides into two-cell stage embryos. Both blastomeres were co-injected with 1 ng myc-tagged Pontin or Reptin mRNA and 4.3 ng of the indicated morpholinos, PMo: antisense Pontin, RMo: antisense Reptin, CoMo: corresponding control morpholino, -Mo: without morpholino. Upper panel shows an immunoblot of embryo lysates stained with 9E10 antibody for the exogenous Pontin and Reptin. Lower panel shows staining for nucleoplasm (NP) used as loading control. (B) Quantification of PH3 positive nuclei in stage 10.5 and 12.5 embryos co-injected at the 2-cell stage with the indicated morpholino oligonucleotides and GFP mRNA. Values are given in relation to the non-injected side. The percentage of positive nuclei at the non-injected side was set to 1. ** *p*-value in student *t*-test < 0.005, * *p*-value in student *t*-test > 0.02. (C) Phenotype of embryos, in which both blastomeres of two-cell stage embryos were injected with Reptin antisense morpholino (RMo: 4.3 ng) alone or with the indicated mRNAs (1 ng RNA each). Phenotypes were analysed at early neurula stage. Left: higher magnification of embryos labelled by white rectangles shown on the right side. Note: lack of the dark line reveals the missing neural tube in embryos with developmental arrest. (D) Percentage of surviving embryos upon injection of 4.3 ng of indicated morpholino oligonucleotides and mRNAs (1 ng each). The numbers of injected embryos are indicated at the bottom.

activated at gastrula stage. Neither overexpression nor depletion of xPontin led to an alteration in the expression of Xbra, chordin and MyoD (Fig. 4). The same results were obtained for xReptin (data not shown).

2.4. The N-termini of pontin and reptin bind to c-Myc

As our mutant analyses revealed that the N-termini of xPontin and xReptin were critical for the mitogenic effect in

both proteins, we sought to identify proteins, which bind to this domain. As shown in Fig. 5A, both N-terminally truncated proteins were still able to interact with β -catenin in GST-pull down experiments. Next we analysed their ability to form homo- and heterodimers. This property is a prerequisite to form hexameric complexes as reported for bacterial helicases (Patel and Picha, 2000). We generated GST-fusion proteins of wild-type and N-terminal truncated xPontin and xReptin and used them in pull-down

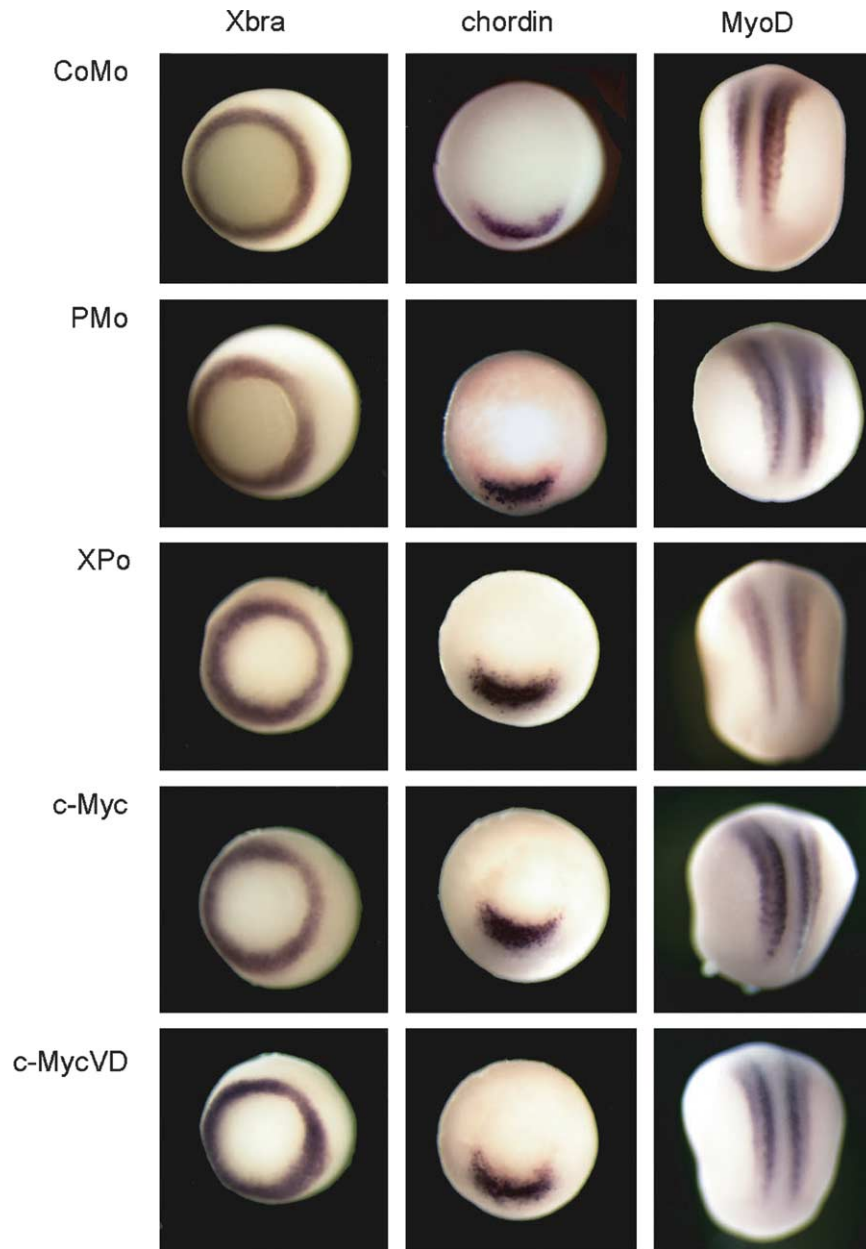


Fig. 4. Neither overexpression of Pontin or c-Myc nor morpholino injections alter the expression of marker genes. 1 ng of the indicated constructs and 4.3 ng PMo were injected into both blastomeres of two-cell stage embryos. None of the marker genes (stage 10 Xbra, stage 11.5 chordin and stage 16 MyoD) was changed. Note: from neurula stage onwards only the surviving PMo injected embryos could be taken for in situ hybridization.

experiments with our myc-tagged protein constructs transiently expressed in HEK 293 cells. As seen in Fig. 5B deletion of the N-terminus did not abolish the formation of homo- or heterodimers.

We also addressed the question whether the N-termini of xPontin and xReptin are required for binding to c-Myc. For this purpose we performed co-immunoprecipitation experiments from HEK 293 cells, which were transfected with flag-tagged murine c-Myc and 9E10 myc-tagged xPontin and xReptin constructs. The 9E10 antibody recognizes part of the human c-Myc sequence present in the myc-tag, which

we used to label our xPontin and xReptin constructs. Importantly, the antibody does not react with murine c-Myc protein so that a clear distinction between murine c-Myc and xPontin or xReptin could be made by their different tags. The co-immunoprecipitation experiments revealed that in contrast to the wild-type proteins, neither xPontin Δ N nor xReptin Δ N co-precipitated with c-Myc from transfected HEK293 cells (Fig. 5C). This result demonstrates that the N-terminal domains of xPontin and xReptin, which are essential to stimulate cell proliferation are specifically required for complex formation with c-Myc.

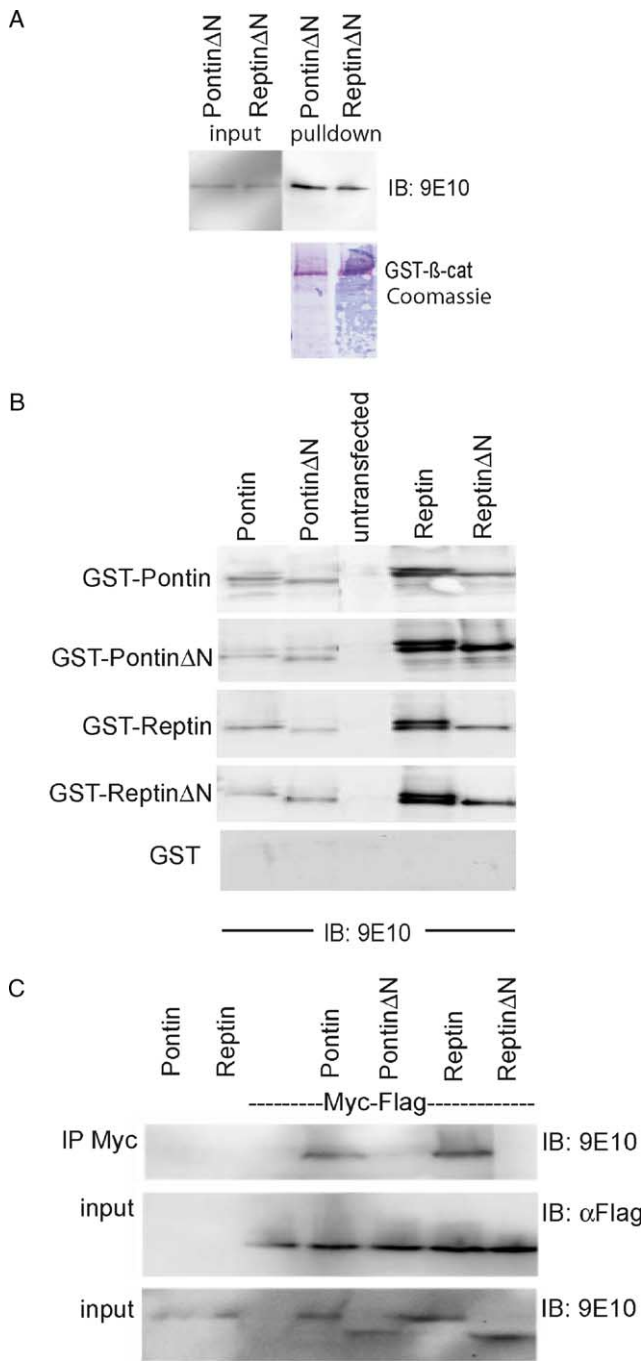


Fig. 5. Deletion of the N-terminus in xPontin and xReptin affects c-Myc binding but not homo- or heterodimerization. (A) GST pull-down assays reveal that N-terminal truncated Pontin and Reptin constructs bind to GST- β -catenin. NOP lysates of transfected HEK 293 cells were incubated with bacterially expressed immobilized GST- β -catenin. Bound proteins were eluted and stained in immunoblots with the 9E10 antibody for the presence of transfected xPontin and xReptin constructs. (B) GST pull-down assays documenting homo- and heterodimerization of full-length xPontin, xReptin and their N-terminally truncated mutants. NOP lysates corresponding to 5×10^6 HEK293 cells transfected with the indicated constructs were incubated with bacterially expressed immobilized GST-Pontin, GST-Pontin Δ N, GST-Reptin, GST-Reptin Δ N or GST alone. Bound proteins were eluted and stained in immunoblots with the 9E10 antibody for the presence of exogenous xPontin and xReptin constructs. The transfected Reptin appears as double band. Most likely, the faster migrating

2.5. xPontin and xReptin are linked to the c-Myc/Miz-1 pathway

We next examined whether xPontin and xReptin indeed display their mitogenic activity through c-Myc. If the function of xPontin and xReptin is coupled to c-Myc, overexpression of c-Myc should mimic the xPontin/xReptin phenotype and c-Myc should be able to rescue the embryonic lethality set by antisense morpholino injections. When mRNA encoding human c-Myc was injected into the dorsal blastomeres, a significant proportion of embryos revealed a convex phenotype indistinguishable from the phenotype of xPontin- or xReptin-injected embryos (Fig. 6A,C). PH3 staining of single sided c-Myc injected embryos revealed an increased number of dividing cells at stage 12.5 (Fig. 2B). Furthermore, injection of mRNA encoding human c-Myc abrogated the embryonic lethality induced by ablation of xPontin or xReptin (Fig. 7A). These results provide strong evidence that xPontin and xReptin regulate cell proliferation through c-Myc.

The c-Myc protein activates transcription as part of a binary complex with its partner protein Max (Eisenman, 2001; Levens, 2003) and represses transcription as part of a trimeric complex that contains the zinc finger protein Miz-1 in addition to Max (Wanzel et al., 2003; Staller et al., 2001). We wanted to elucidate in which of these mechanisms xPontin and xReptin are involved. To answer this question, we used specific components of the different c-Myc pathways to phenocopy the gain-of-function or to rescue the loss-of-function phenotype of xPontin and xReptin. To test whether binding to Miz-1 is required, we used a single point mutant of c-Myc (MycV394D) that binds to Max and activates transcription as wild-type c-Myc, but does not bind to Miz-1 and does not repress transcription (Herold et al., 2002). In contrast to wild-type c-Myc, MycV394D was unable to induce a convex phenotype when injected into the dorsal blastomeres, despite being expressed at equal levels to wild-type c-Myc (Fig. 6A,B,C). When injected together with xPontin or xReptin mRNA, MycV394D reduced the formation of convex embryos, whereas wild-type c-Myc enhanced the phenotype induced by xPontin or xReptin (Fig. 6C). Consistently, MycV394D did not significantly stimulate cell proliferation (Fig. 2B). Finally, MycV394D was unable to rescue the embryonic lethality induced by ablation of xPontin or xReptin, in contrast to wild-type c-Myc (Fig. 7A).

band is due to protein degradation. (C) Binding of Pontin and Reptin to Myc requires the amino terminus. Shown are co-immunoprecipitation experiments from HEK293 cells transfected with flag-tagged murine Myc and 9E10 myc-tagged xPontin and xReptin constructs as indicated. Note: 9E10 specifically recognizes human (myc-tag) but not murine Myc protein. Lysates were precipitated with α -flag-antibody ('IP Myc'); immunoblots were probed with the 9E10 antibody (IB 9E10) for the presence of exogenous xPontin and xReptin constructs or with α -flag-antibody (IB α flag) for exogenous c-Myc. Co-immunoprecipitations were done according to (Herold et al., 2002).

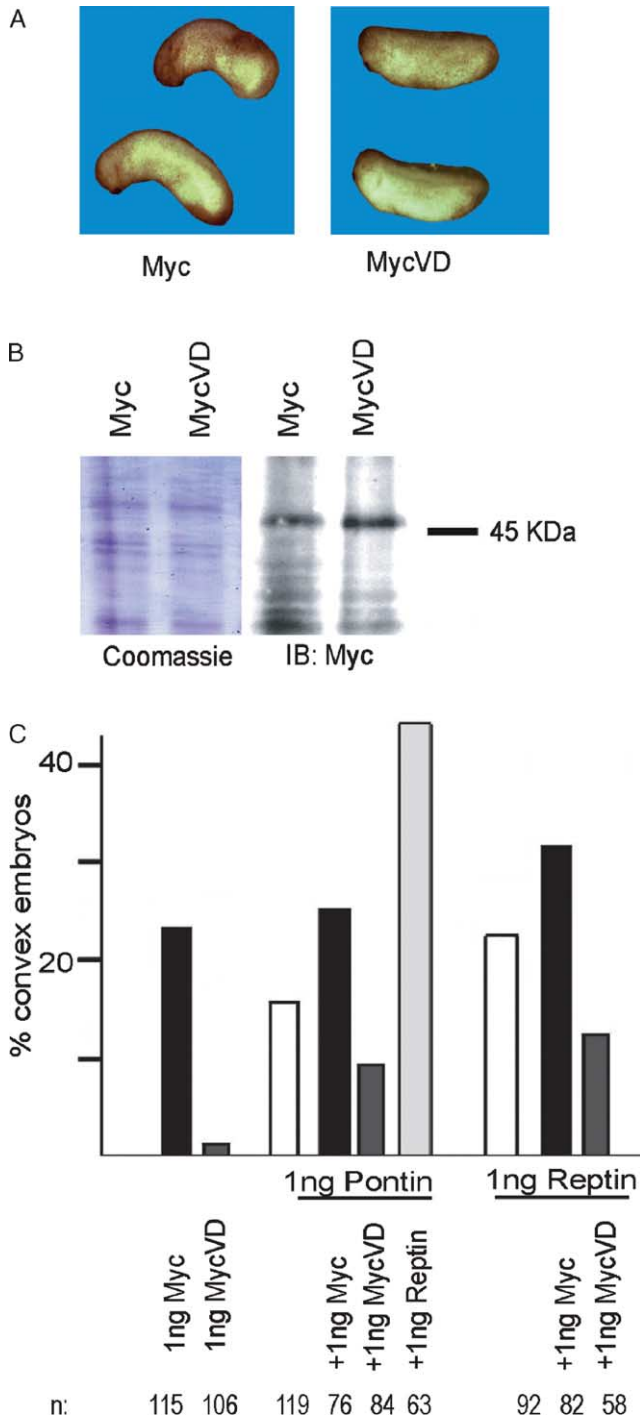


Fig. 6. c-Myc but not the c-MycV394D mutant deficient in Miz-1 binding phenocopies xPontin and xReptin. (A) Representative examples of embryos dorsally injected with 1 ng mRNA encoding either human c-Myc or human c-MycV394D (MycVD). (B) Coomassie stained gel and an immunoblot against human c-Myc-protein. The gel was loaded with lysates of injected embryos, documenting equal expression of the constructs. (C) Percentage of embryos showing a convex phenotype after injection of 1 ng RNA encoding the indicated proteins into both dorsal blastomeres of 4-cell stage embryos. Co-injection of c-Myc either with xPontin or xReptin increased the number of convex shaped embryos while c-MycVD decreased it. Note: xPontin and xReptin co-injection shows an additive effect compared to single injections either of xPontin or xReptin.

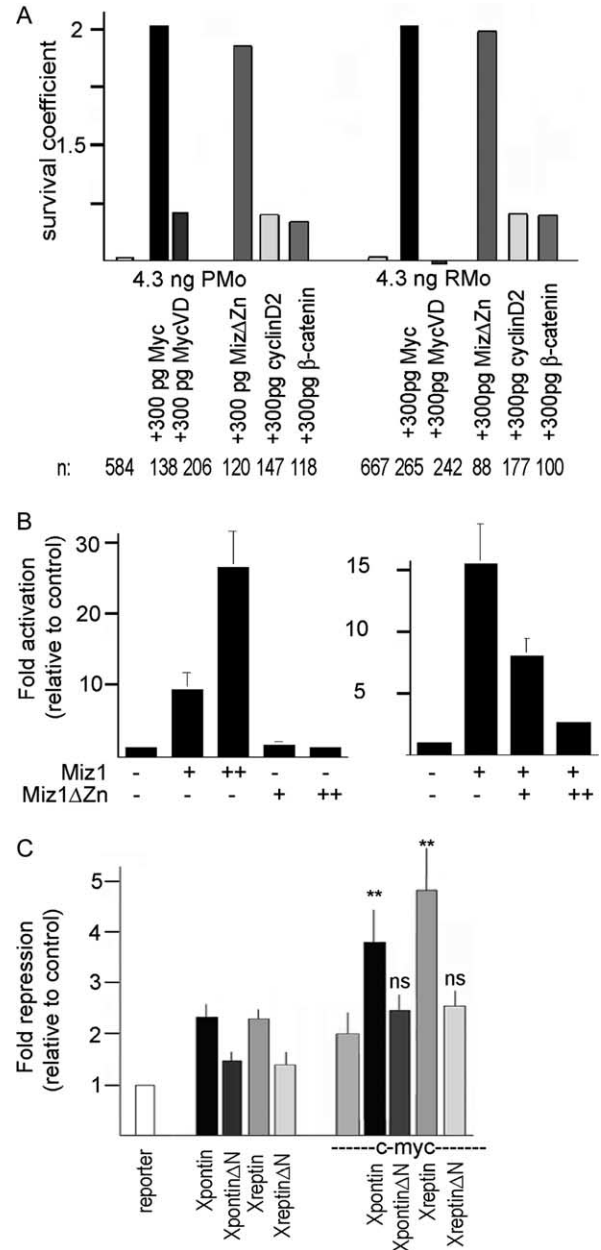


Fig. 7. Pontin and Reptin are co-repressors of c-Myc/Miz-1. c-Myc and dominant negative Miz-1 but not the c-MycV394D mutant, cyclin D2 or β-catenin rescue morpholino induced developmental arrest. Quantification of embryonic survival upon injection of the indicated constructs into both blastomeres of two-cell stage embryos. Phenotypes were analysed at early neurula stage. The survival coefficient is defined as the percentage of surviving embryos co-injected with the indicated constructs relative to morpholino-alone injected embryos of the same egg batch. Thus, the survival coefficient normalizes the different quality of egg batches so that a direct comparison of the injections is possible. Depending on the egg batch, PoMo led in 29–70% of the injected embryos to gastrula arrest, ReMo in 33–71%. The numbers of injected embryos are indicated at the bottom. (B) Miz1ΔZn is a dominant negative mutant of Miz-1. Shown are transient transfection assays in HeLa cells using the indicated CMV-based expression constructs. The p21 promoter was used as a reporter. The left panel documents the inability of Miz1ΔZn to activate the p21 reporter; the right panel documents the dominant negative activity towards wtMiz-1. (C) Xpontin and xReptin but not their N-terminal mutants enhance p21 repression in Hek293 cells in normal and co-transfected c-Myc background.

Since expression of c-Myc inhibits transactivation by Miz-1, we argue that xPontin and xReptin are co-repressors for c-Myc and that the lethality induced by ablation of either xPontin or xReptin is due to the growth-suppressive activity of Miz-1. To test this possibility, we generated a dominant negative construct of Miz1, Miz1 Δ Zn that lacks the Zn finger (DNA binding) domain. In contrast to full-length protein, Miz1 Δ Zn did not activate the p21 promoter and when co-transfected with the full-length form it was able to block Miz-1 induced p21 promoter activation (Fig. 7B). Expression of Miz1 Δ Zn in xPontin or xReptin depleted embryos (Fig. 7A) led to their survival demonstrating that inhibition of Miz-1 transactivation is sufficient to bypass the requirement for xPontin or xReptin.

Since the MycV394D mutant, which still can bind Max, fails to behave like wild-type c-Myc in our functional assays we suggest that xPontin and xReptin are not involved in the activating c-Myc pathway. This was further confirmed by expression of cyclinD2 in xPontin or xReptin depleted embryos, which did not abolish embryonic lethality (Fig. 7A). Also β -catenin RNA injections did not rescue the loss-of-function phenotype, which excludes a putative contribution of Wnt/ β -catenin signalling to the mitogenic effect of xPontin and xReptin (Fig. 7A).

From these results we conclude that xPontin and xReptin promote cell proliferation in *Xenopus* embryos as co-factors in the c-Myc/Miz-1 pathway. We confirmed the co-repressor function of xPontin and xReptin in p21 promoter reporter assay using HEK 293 cells. As expected, full-length but not N-terminal truncated xPontin and xReptin repress p21 reporter activity in normal and in c-Myc increased background (Fig. 7C).

2.6. *Xenopus Miz-1 is expressed in early development*

c-Myc, xPontin and xReptin are maternally provided and expressed throughout early development (Houdry et al., 1988; Vrız et al., 1989; Bellmeyer et al., 2003; Etard et al., 2000). To obtain further evidence that Miz-1 can physiologically collaborate with c-Myc, xPontin and xReptin, we cloned a partial cDNA of *Xenopus* Miz-1 and analysed by RT-PCR and in situ hybridisation whether it is also expressed in early embryogenesis. Sequence alignment revealed an overall identity of 58% between the human and the predicted *Xenopus* Miz-1 protein including the highly conserved Zn-finger domain with 84% identity. Miz-1 like c-Myc, xPontin and xReptin is maternally provided and localized in the animal hemisphere until blastula stage (supplementary Fig. S3). Thus, all components are present at the same stages underlining the physiological significance of the co-repressor function of xPontin and xReptin in the c-Myc/Miz-1 complex controlling cell proliferation early in *Xenopus* development.

3. Discussion

Here we describe the function of xPontin and xReptin in regulating cell proliferation by acting as co-repressors in the c-Myc/Miz-1 pathway early in development. Knockdown of either xPontin or of xReptin results in embryonic lethality, which is abrogated by inhibition of Miz-1 transactivation. The latter is achieved either by increasing c-Myc, which blocks the transcription factor Miz-1 or by expression of a dominant-negative Miz-1 construct that lacks the zinc finger domain. In contrast, the MycV394D mutant, which interacts with Max but not with Miz-1 is not able to rescue embryonic lethality. We suppose that reduction of the co-repressors xPontin and xReptin in the c-Myc/Miz pathway leads to decrease in cell proliferation and developmental arrest, because knockdown of either protein resulted in a substantial reduction of PH3 positive cells. Conversely, overexpression of xPontin or xReptin increased the number of mitotic cells and resulted in a bending of embryos.

Earlier reports describe critical periods and regions of cell proliferation in *Xenopus* development. When cell division was blocked at blastula stage (7–8) developmental arrest was observed after gastrulation movement begun (Cooke, 1973). During gastrulation inhibition of cell division has apart from delay in development little effect on further development (Cooke, 1973; Audic et al., 2001). Variations in the strength of the phenotype between knockdowns and dominant negative expression of individual cell cycle regulators are explained by different amounts of maternally provided protein and differences in post-translational activation (Hartley et al., 1997; Audic et al., 2001). For example, cell proliferation during gastrulation is blocked by Wee-1, an inhibitor of CyclinB/Cdc2, which is activated by phosphorylation at stage 10. The protein is present throughout early development (Murakami et al., 2004). This supports previous findings that mitotic cells are found in the ectoderm and endoderm but not in the involuting mesoderm (Saka and Smith, 2001). These observations indicate that cell division might be required for epiboly of the ectoderm and not for convergent extension of the axial mesoderm during gastrulation. We think that our results support the previous data: overexpression of xPontin and xReptin increases cell proliferation in all germ layers from stage 12.5 onwards. This would explain the bending of embryos when xPontin or xReptin is overexpressed in the dorsal or ventral side of the embryo. Depletion of xPontin and xReptin, however, affects cell proliferation much earlier; a significant decrease in mitotic cells is already observed when the mesoderm starts involution (stage 10.5). This might result in morphogenetic defects as Cooke (1973) has described when cell division is blocked before gastrulation. Apart from the mitogenic effect we did not observe alterations in the expression of mesodermal markers or Wnt/ β -catenin target genes, which does not exclude that xPontin and xReptin may have additional functions in still unknown processes.

Importantly, the N-terminal deletion mutants of xPontin and xReptin that bind β -catenin but not c-Myc clearly point to a c-Myc dependent mechanism because these mutants have lost the mitogenic activity.

Remarkably, we did not observe that either the Walker A or Walker B motif in xPontin or xReptin was crucial in stimulating cell proliferation because corresponding deletion mutants behaved as the wild-type proteins. Previous reports showed that a missense mutation in the Walker B motif of TIP 49 resulted in loss of c-Myc induced oncogenic transformation (Wood et al., 2000) and increased c-Myc dependent apoptosis (Dugan et al., 2002). The latter indicates to an enhanced c-Myc activity of the missense mutation in their system. The same missense mutation in the bacterial RuvB has been shown to abolish ATP hydrolysis (Mezard et al., 1997). Consistent with our functional data Wood et al. (2000) reported that neither deletion of the Walker A or the Walker B motif affected the interaction with c-Myc. We mapped the N-terminus in both proteins as c-Myc interaction domain and demonstrated its functional relevance. Although our N-terminal truncations include the Walker A motif, we suggest that the N-terminal region in front of the Walker A motif is important for c-Myc binding because overexpression of the Walker A deletion mutants results in bent embryos as the wild-type proteins (Fig. 1C). With our work, we extend former deletion analyses by Wood et al. (2000) who observed a reduced c-Myc binding of an internal deletion mutant of TIP49 covering a stretch of 51 aa between the Walker A and Walker B motifs.

In cooperation with c-Myc/Miz-1 xPontin and xReptin exhibit new properties because they act similar and additive in stimulating cell proliferation. Gain- and loss-of-function of either protein had the same effect, bending or embryonic lethality. Both proteins repress p21 promoter reporter activity in HEK 293 cells. Either protein is required in early development (Fig. 3D). Moreover, they act additive when both RNAs are co-injected (Fig. 6C). Taken together, xPontin and xReptin in the c-Myc/Miz-1 pathway behave differently compared to their role in Wnt/ β -catenin signaling in, which they act antagonistically (Bauer et al., 2000; Rottbauer et al., 2002).

Knockdown of either protein in *Xenopus* revealed that they are essential during gastrula stage. This time point might display the transition from maternally provided to zygotically derived protein pools. The amount of maternally provided protein may differ between xPontin and xReptin and c-Myc, since translational repression of c-Myc did not cause a similar developmental arrest (Bellmeyer et al., 2003).

Together, our findings suggest that xPontin and xReptin have mechanistically distinct roles in different transcription complexes. They act as co-repressor in the c-Myc/Miz-1 pathways to control cell proliferation. Since these proteins are highly conserved, we suppose that dysfunction of the Pontin/Reptin-Myc/Miz-1 complex may have multiple consequences in developmental differentiation processes as well as for tumorigenesis.

4. Methods

4.1. Plasmids, RNA transcription and RT-PCR

Deletions and mutations in *Xenopus* Pontin and Reptin (Etard et al., 2000) were inserted by PCR strategies (primer sequences are available upon request) and C-terminally fused to six copies of the myc(9E10)-epitope. Wild-type and N-terminal truncations were also fused with GST in pGEX3. GST- β -catenin, hPontin and hReptin were described elsewhere (Bauer et al., 2000). The p21 luciferase construct was described in Herold et al. (2002), the CMV- β -galactosidase construct is described elsewhere (Gradl et al., 2002). Dominant negative Miz1 (Miz1 Δ Zn), human Myc-V394D mutant (Staller et al., 2001) and human cyclin D2 (Bouchard et al., 2001) were subcloned in pCS2+, β -catenin for injections was as in Behrens et al. (1996).

Capped RNAs were synthesized in vitro using the Message Machine Kit (Ambion). RT-PCR was done according to Etard et al., 2000, using as primers for *siamois* forw. 5'-GGG GAG AGT GGA AAG TGG TTG-3' and rev. 5'-CTC CAG CCA CCA GTA CCA GAT C-3', for *Xnr3* forw. 5'-ATC TCT TCA TGG TGC CTC AGG-3' and rev. 5'-TCC ACT TGT GCA GTT CCA CAG-3' and for *Xmiz-1* forw. 5'-GCC TGG TGA GTC TCC TGA AC-3' and rev. 5'-GGG AAA CCT GAA AGC CCA CC-3'.

4.2. Microinjection and cultivation of *Xenopus laevis* embryos

For loss of function experiments, morpholino oligonucleotides and in vitro transcribed mRNA were injected into one or two blastomeres of *Xenopus laevis* two-cell stage embryos. Sequences of the antisense morpholino oligonucleotides are for *Xenopus* Pontin: 5'-c atg aaa atc gag gag gtg aag agc-3', corresponding control: 5'-c atg taa atg gag gac gtg atg agc-3'; *Xenopus* Reptin: 5'-g aag ata tag tgc agc atg gca acc-3', corresponding control: 5'-g aag aat tac tgc agg atg gga acc-3'. For co-injections with β -catenin mRNA, both ventral blastomeres of 4-cell stage embryos were injected. For gain of function experiments, both dorsal blastomeres were injected. Embryos were cultivated as described (Kühl et al., 1996). In general 1 ng RNA of each construct was injected. If less than 1 ng RNA was applied it is mentioned in the Figure.

Treatment with hydroxyurea and aphidicolin was carried out by adding hydroxyurea in a final concentration of 20 mM and aphidicolin in a final concentration of 150 μ M to devitelinized stage 10 embryos (Hardcastle and Papalopulu, 2000). Embryos were kept in this solution until fixation.

4.3. Immunostaining

Stage 10.5 and 12.5 embryos were fixed for 1 h in 4% PFA in 100 mM NaCl and 100 mM HEPES pH7.4 and equilibrated

over night at -20°C in Dent's (20% DMSO, 80% Methanol). After washing for 30 min with 100 mM NaCl in 100 mM Tris/HCl pH 7.4 and over night in 15% gelatine/15% saccharose embryos were embedded in 25% gelatine/15% saccharose over night and cut with a vibratome (Leica) in sections of 16 μm thickness. These sections were blocked for 5 min with 0.1% Triton, 0.1% saponine and 10% BSA in APBS/ Ca^{2+} (103 mM NaCl, 2.7 mM KCl, 0.15 mM KH_2PO_4 , 0.7 mM NH_2PO_4 , 2 mM CaCl_2 , pH 7.5), incubated overnight with monoclonal α -myc(9E10)-epitope and polyclonal anti phosphohistone H3 (Upstate Biotechnology) antibodies. Visualization was performed with Cy3- or Cy2-labeled goat anti-mouse or goat anti-rabbit antibodies. After counterstaining the nuclei with DAPI, cells were embedded in Elvanol and analyzed by fluorescence microscopy (Improvision software, Leica).

4.4. In situ hybridizations

Whole-mount in situ hybridization was performed according to previously described procedures (Gawantka et al., 1995). Localization of mRNA was visualized using anti-digoxigenin antibodies conjugated to alkaline phosphatase, followed by incubation with nitro blue tetrazolium (NBT) and 5-bromo 4-chloro 3-indolyl phosphate (BCIP). Images were captured on a Leica MZFLIII microscope using a digital camera (Qimaging) and Improvision software (Openlab). In situ probes for the detection of Xbra (Smith et al., 1991), MyoD (Rupp and Weintraub, 1991) and chordin (Sasai et al., 1995) were as described the probe for c-Myc was kindly provided by K. Henningfeld. Based on EST sequence data, a 1200 bp XMiz-1 fragment was amplified and subcloned in pGEMT.

4.5. GST-pulldowns and immunoblotting

Expression of recombinant proteins in BL21D3 cells was induced by adding 1 mM IPTG at $\text{OD}_{600}=0.8$. After incubation for 4 h at room temperature, proteins were extracted by sonication in PBS. For pulldown experiments NOP lysates (150 mM NaCl, 10 mM Tris/HCl pH 7.8, 1 mM MgCl_2 , 0.75 mM CaCl_2 , 2% Nonidet P40) corresponding to 5×10^6 transfected HEK293 cells were incubated with immobilized fusion proteins according to previously described protocols (Bauer et al., 2000). Bound proteins were separated on a 7.5% SDS PAGE, transferred onto nitrocellulose and incubated with the 9E10 antibody. Visualization was performed using peroxidase coupled secondary antibody and ECL-plus substrate (Amersham). For determining the expression of the injected *Xenopus* Pontin/Reptin and human Myc constructs, NOP-lysates of injected embryos corresponding to one half embryo were used for immunoblotting.

4.6. Cell culture, promoter reporter assays and immunoprecipitation

For promoter reporter assays, HEK293 cells were co-transfected with 4 μg Pontin or Reptin constructs together with 1 μg murine c-Myc, 4 μg p21-luciferase and 1 μg CMV- β -galactosidase. Reporter assays were done as previously described (Gradl et al., 2002). To characterize the Miz-1 ΔZn mutant, HeLa cells were co-transfected with p21-luciferase together with 1–2 μg wild-type Miz-1 and Miz-1 ΔZn . For immunoprecipitations 4 μg murine Myc-flag were co-transfected with the myc-tagged *Xenopus* Pontin or Reptin constructs. 150 μl RIPA lysate was pre-cleared with 30 μl protein A sepharose (Amersham) and incubated for 1 h with 10 μg of 9E10 or anti-flag antibody (Sigma) together with 2% BSA. Precipitated proteins were eluted with 10 μl SDS sample buffer and analysed by immunoblotting using 9E10 antibody for staining *Xenopus* Pontin and Reptin constructs.

Acknowledgements

This study was supported by grants from the Deutsche Forschungsgemeinschaft (DFG) to DW. We would like to thank M.A. Wood for flag tagged murine c-Myc, Steffi Herold for testing the Miz ΔZn mutant in HeLa cells (Fig. 6 B) and Ariane Tomsche and Ira Röder for technical assistance.

Appendix A. Supplementary Material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.mod.2004.11.010

References

- Audic, Y., Boyle, B., Slevin, M., Hartley, R.S., 2001. Cyclin E morpholino delays embryogenesis in *Xenopus*. *Genesis* 30, 107–109.
- Bauer, A., Chauvet, S., Huber, O., Usseglio, F., Rothbächer, U., Aragnol, D., et al., 2000. Pontin52 and reptin52 function as antagonistic regulators of β -catenin signalling activity. *Eur. Mol. Biol. Org. J.* 19, 6121–6130.
- Behrens, J., von Kries, J.P., Kühl, M., Bruhn, L., Wedlich, D., Grosschedl, R., Birchmeier, W., 1996. Functional interaction of β -catenin with the transcription factor LEF-1. *Nature* 382, 638–642.
- Bellmeyer, A., Karase, J., Lindgren, J., LaBonne, C., 2003. The protooncogene c-myc is an essential regulator of neural crest formation in *Xenopus*. *Dev. Cell* 4, 827–839.
- Bouchard, C., Dittrich, O., Kiermaier, A., Dohmann, K., Menkel, A., Eilers, M., Luscher, B., 2001. Regulation of cyclin D2 gene expression by Myc/Max/Mad network: Myc-dependent TRRAP recruitment and histone acetylation at the cyclin D2 promoter. *Genes Dev.* 15, 2042–2047.
- Bouchard, C., Marquardt, J., Bras, A., Medema, R.H., Eilers, M., 2004. Myc-induced proliferation and transformation require Akt-mediated phosphorylation of FoxO proteins. *Eur. Mol. Biol. Org. J.* 23 (14), 2830–2840.

- Cooke, J., 1973. Properties of the primary organization field in the embryo of *Xenopus laevis*. *J. Embryol. Exp. Morphol.* 30, 49–62.
- Dugan, A.D., Wood, M.A., Cole, M.D., 2002. TIP49, but not TRAP, modulates c-Myc and E2F1 dependent apoptosis. *Oncogene* 21, 5835–5843.
- Eisenman, R.N., 2001. Deconstructing Myc. *Genes Dev.* 15, 2023–2030.
- Etard, C., Wedlich, D., Bauer, A., Huber, O., Kuhl, M., 2000. Expression of *Xenopus* homologs of the β -catenin binding protein pontin52. *Mech. Dev.* 94, 219–222.
- Gawcastle, V., Delius, H., Hirschfeld, K., Blumenstock, C., Niehrs, C., 1995. Antagonizing the Spemann organizer: role of the homeobox gene *Xvent-1*. *Eur. Mol. Biol. Org. J.* 14, 6268–6279.
- Grädl, D., König, A., Wedlich, D., 2002. Functional diversity of *Xenopus* Lymphoid enhancer factor/T-cell factor transcription factors relies on combinations of activating and repressing elements. *J. Biol. Chem.* 277, 14159–14171.
- Hardcastle, Z., Pappalopulu, N., 2000. Distinct effects of XBF-1 in regulating the cell cycle inhibitor p27(XIC1) and imparting a neural fate. *Development.* 127, 1303–1314.
- Hartley, R., Sible, J., Lewellyn, L., Maller, J.L., 1997. A Role for Cyclin E/Cdk2 in the Timing of the Midblastula transition in *Xenopus* embryos. *Dev. Biol.* 188, 312–321.
- Herold, S., Wanzel, M., Beuger, V., Frohme, C., Beul, D., Hillukalla, T., et al., 2002. Negative regulation of the mammalian UV response by Myc through association with MIZ-1. *Mol. Cell* 10, 509–521.
- Hishida, T., Iwasaki, H., Yagi, T., Shinagawa, H., 1999. Role of walker motif A of RuvB protein in promoting branch migration of holliday junctions. Walker motif mutations affect Atp binding, Atp hydrolyzing, and DNA binding activities of Ruvb. *J. Biol. Chem.* 274, 25335–25342.
- Houdry, J., Brulfert, A., Gusse, M., Schoevaert, D., Taylor, M., Mechali, M., 1988. Localization of c-myc expression during oogenesis and embryonic development in *Xenopus laevis*. *Development* 104, 631–641.
- Jonsson, Z.O., Dhar, S.K., Narlikar, G.J., Auty, R., Wagle, N., Pellman, D., et al., 2001. Rvb1p and Rvb2p are essential components of a chromatin remodelling complex that regulates transcription of over 5% of yeast genes. *J. Biol. Chem.* 276, 16279–16288.
- Kanemaki, M., Makino, Y., Yoshida, T., Kishimoto, T., Koga, A., Yamamoto, K., et al., 1997. Molecular Cloning of a rat 49-kDa TBP-interacting protein (TIP49) that is highly homologous to the bacterial RuvB. *Mol. Biophys. Res. Com.* 235, 64–68.
- Kanemaki, M., Kurokawa, Y., Matsu-ura, T., Makino, Y., Masani, A., Okazaki, K., et al., 1999. TIP49b, a New RuvB-like DNA Helicase, is included in a complex together with another RuvB-like DNA helicase, TIP49a. *J. Biol. Chem.* 274, 22437–22444.
- Kühl, M., Finnemann, S., Binder, O., Wedlich, D., 1996. Dominant negative expression of a cytoplasmically deleted mutant of XB/U-cadherin disturbs mesoderm migration during gastrulation in *Xenopus laevis*. *Mech. Dev.* 54, 71–82.
- Levens, D., 2003. Reconstructing Myc. *Genes Dev.* 17, 1071–1077.
- Lim, C.R., Kimata, Y., Ohdate, H., Kokubo, T., Kikuchi, N., Horigome, T., Kohno, K., 2000. The *Saccharomyces cerevisiae* RuvB-like protein, Tih2p, is required for cell cycle progression and RNA polymerase II-directed transcription. *J. Biol. Chem.* 275, 22409–22417.
- Makino, Y., Kanemaki, M., Kurokawa, Y., Koji, T., Tamura, T.-A., 1999. A rat RuvB-like protein, TIP49a, is a germ cell-enriched novel DNA helicase. *J. Biol. Chem.* 274, 15329–15335.
- Mezard, C., Davies, A.A., Stasiak, A., West, S.C., 1997. Biochemical properties of RuvB D113N: A mutation in helicase motif II of the RuvB hexamer affects DNA binding and ATPase activities. *J. Mol. Biol.* 271, 704–717.
- Murakami, M.S., Moody, S.A., Dear, I.O., Morrison, D.K., 2004. Morphogenesis during *Xenopus* gastrulation requires Wee1-mediated inhibition of cell proliferation. *Development* 131, 571–580.
- Nieuwkoop, P.D., Faber, J., 1967. Normal table of *Xenopus laevis*. Elsevier North-Holland Biochemical Press, Amsterdam.
- Patel, S.S., Picha, K.M., 2000. Structure and function of hexameric helicases. *Annu. Rev. Biochem.* 69, 651–697.
- Rottbauer, W., Saurin, A.J., Lickert, H., Shen, X., Burns, C.G., Wo, Z.G., et al., 2002. Reptin and pontin antagonistically regulate heart growth in zebrafish embryos. *Cell* 111, 661–672.
- Rupp, R.A., Weintraub, H., 1991. Ubiquitous MyoD transcription at the midblastula transition precedes induction-dependent MyoD expression in presumptive mesoderm of *X. laevis*. *Cell* 65, 927–937.
- Saka, Y., Smith, J.C., 2001. Spatial and temporal patterns of cell division during early *xenopus* embryogenesis. *Dev. Biol.* 229, 307–318.
- Sasai, Y., Lu, B., Steinbeisser, H., de Robertis, E.M., 1995. Regulation of neural induction by the Chd and Bmp-4 antagonistic patterning signals in *Xenopus*. *Nature* 376, 333–336.
- Seoane, J., Pouppnot, C., Staller, P., Schader, M., Eilers, M., Massague, J., 2001. TGF β influences Myc Miz-1 and Smad to control the CDK inhibitor p15ink4b. *Nature Cell Biol.* 3, 400–408.
- Seoane, J., Le, H.V., Massague, J., 2002. Myc suppression of the p21(Cip) Cdk inhibitor influences the outcome of the p53 response to DNA damage. *Nature* 419, 729–734.
- Shinagawa, H., Iwasaki, H., 1996. Processing the holliday junction in homologous recombination. *Trends Biochem. Sci.* 21, 107–111.
- Smith, J.C., Price, B.M., Green, J.B., Weigel, D., Herrmann, B.G., 1991. Expression of a *Xenopus* homolog of Brachyury (T) is an immediate-early response to mesoderm induction. *Cell* 67, 79–87.
- Staller, P., Peukert, K., Kiermeier, A., Seoanne, J., Lukas, J., Karsunky, H., et al., 2001. Repression of p15ink4b expression by myc through association with miz-1. *Nature Cell Biol.* 3, 392–396.
- Vriz, S., Taylor, M., Mechali, M., 1989. Differential expression of two *Xenopus* c-myc proto-oncogenes during development. *Eur. Mol. Biol. Org. J.* 8, 4091–4097.
- Wanzel, M., Herold, S., Eilers, M., 2003. Transcriptional repression by Myc. *Trends Cell Biol.* 13, 146–150.
- Wood, M.A., McMahon, S.B., Cole, M.D., 2000. An ATPase/helicase complex is an essential cofactor for oncogenic transformation by c-Myc. *Mol. Cell* 5, 321–330.